Vietnam Journal of Science and Technology ***56*** *(4A) (2018) 1-12*

Mini Review

APPLICATION OF IMAGE-BASED HIGH CONTENT ANALYSIS FOR THE SCREENING OF BIOACTIVE NATURAL-PRODUCTS

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Received: 20/9/2018; Accepted for publication: 14 October 2018

ABSTRACT

The phenotypic changes in target cell induced by an exogenous agent, e.g. biologically active chemical, can be detected by labelling selected organelles with fluorescent dyes and observed under the high-throughput microscope. The changes of known biological targets (e.g. nucleus, proteins, mitochondria, microtubules, etc.) induced by a compound of interest allow predicting its mechanisms of action. High-throughput microscopy with capability of multiparametric imaging in combination with automated image-analysis is referred to as high-content analysis or high-content screening (HCS). Recently, HCS plays an increasingly important role for a large-scale screening of small molecules for several aspects including anti-cancer, anti-inflammatory activities, disease-related biomarkers and *in vitro* toxicity testing, and so on. As an introduction, this short review covers the concepts of HCS and its application in screening of biologically active natural products whose molecular targets could be identified through such approaches.

Keywords: cell-based assay, high-content screening, automated microscope, imaging technology, bioactive natural-products.

1. INTRODUCTION

Natural products have largely been considered as an extraordinary valuable platform for the discovery of new drugs against diverse pathologies and a tool for the characterization of novel therapeutic targets as well. Despite recent advances in chromatographic and spectroscopic techniques and the rich tradition of the use of herbal medicines, still little effort of natural

product chemists has been directed towards isolating the bioactive chemical constituents from the natural sources. Reasons for this fact are related to the lack of expertise and infrastructure for biological screening, high costs as well as time consuming if samples must be sent to overseas laboratories. It is therefore highly desirable to develop in-house bioassays in institutes for chemical research which are inexpensive, rapid and do not require the chemists a specialized knowledge of biology or pharmacology [1-3]. The bioassay laboratories at Vietnam Academy of Science and Technology (VAST) had therefore initially established the "bench-top" bioassays since the 1990s. In recent years, some VAST's laboratories have been further provided with essential and modern devices enabling development of more up-to-date methods to reduce subjective interpretation. Accordingly, this overview looks at the current screening method utilizing a modern technique, a high content screening (HCS), which is designed for various applications, not only in life science research but also in drug discovery to screen for hit compounds that impact cellular functions such as arrested cell-cycle, apoptosis, translocation, etc.

2. GENERAL CONCERPT OF HCS

The phenotypic changes in target cell induced by a bioactive compound can be detected by labelling selected organelles with fluorescent dyes and observed under the high-throughput microscope. A comparison of the cellular phenotype induced by a compound of interest with the phenotypes of reference compounds with known cellular targets allows predicting its mode of action [4, 5].

Figure 1. A high content screening station (Olympus scan^R)
at Institute of Natural Products Chemistry (INPC-VAST).

HCS is a relatively new approach applied to detect changes at a cell and/or subcellular level with simultaneous readout of several parameters by using a high-throughput microscopy in combination with automated multiparametric-image analysis. Multiwavelength fluorescence imaging is the key technology for HCS platforms. This technology enables the detection of many cellular features in a single preparation, and imaging software can extract both qualitative and quantitative data from a variety of fluorescent parameters, e.g., fluorescence intensity, cellular and subcellular morphometrics, and a total count of features. Such HCS platforms acquire wavelength channels either sequentially or in parallel. With sequential acquisition, each fluorophore is separately excited and detected on a single monochrome charged coupled digital (CCD) camera. Most HCS systems are nowadays equipped with filter sets for the most common

fluorescent probes and can distinguish up to four or eight labels in a single preparation with minimal crosstalk between channels. Fluorescent microscopy imaging assays are therefore referred to as "high content" in comparison to most other cell-based assays that only generate single-parameter readouts [6-8].

In fact, HCS can be applied in two contexts. In a HCS experimental and data evaluation are more multiplex, thus going along with a lower throughput (smaller number) of tested compounds. The visual assessment of phenotypical changes of cells can add a significant measure to target- or mode of action predictions of uncharacterized compounds. Inversely, HCS can also be used as other high-throughput screening (HTS) methods since it has functioned well for rapidly and effectively screening large compound libraries of up to thousands of small molecules per day. In this context, a fixed endpoint and smaller number of parameters are often selected to facilitate automation (robotization), reduce procedural steps and data storage [4, 9]. Moreover, miniaturizing the imaging readout to a 96-, 384- or 1536-well format is advisable, thus a small volume of cells could be established. Nowadays a number of HCS platforms have been developed with a variety of design concepts based on the instruments versatility and overall cost, such as IN CELL Analyzer systems (GE Healthcare, Piscataway, NJ), Opera systems (Perkin Elmer, Waltham, MA), ImageXpress® Micro/Ultra (Molecular Devices, Sunnyvale, CA), scan^R (Olympus Europa SE & Co.KG, Hamburg, DE), CellInsight™ CX (Thermo Fisher Scientific, Waltham, MA), etc. The growing availability of HCS infrastructure within research institutions has increasingly spurred interest in HCS applications among academic investigators.

3. ESSENTIALS AND PERSPECTIVES OF HCS

In general, HCS has been developed to meet the needs for flexible applications in both life science research and drug discovery. Screening steps in early drug discovery traditionally used primarily homogeneous assays because they were relatively simple "mix and read" assays. This approach was compatible with the prevailing view in the 1990s that stressed fast measurements on a growing list of targets with large numbers of compounds. Despite changing strategies in natural product research, in fact, the productivity of the whole pharmaceutical industry has decreased over the last decades leading to the exploitation of modern high-throughput technology to make changes in hit-to-lead stage. An alternative approach to easing key bottlenecks in the drug discovery process was suggested, based on HCS [11]. This image-based assay has opened the opportunity to perform drug discovery, not just on a preselected target, but to screen for compounds that impact cellular functions such as cell cycle, translocation, apoptosis, and so on [12]. A major bottleneck in the exploration of natural products as tools for chemical biology research and as lead structures for therapeutic applications is the identification of their mode of action on a molecular and cellular level. HCS method could be utilized to quickly identify a compound's cellular target if this belongs to a known mode of action class. The compound can then be directed to specific target-based biochemical and/or biophysical assays to verify the hypothesis generated by HCA [4]. For compound profiles that cannot be matched, direct identification techniques, e.g. genetic screens or pulldown probes, could be further applied. Such a systematic approach may render the assignment of a biological profile for natural products more efficient and increase their value for life sciences significantly.

In recent years, technological developments in various fields such as HCS analysis software, *in vitro* cellular models, genome engineering, chemoinformatic tools and phenotypic compound libraries provided the investigators significant capabilities to measure various biological out- puts in the most physiopathologically relevant *in vitro* models. According to

Dorval *et al.* [10], however, to fully benefit from the of HCS's capabilities for either target or drug discovery, it is critical to select the appropriate projects, taking into account (i) the level of understanding of the molecular processes involved in the biology of interest, (ii) the availability of a physiopathologically relevant *in vitro* models adapted to medium and large scale screening campaigns, (iii) the ability to select and measure the most appropriate and diversified phenotypic and functional features to provide a sufficient level of agnosticism and finally, (iv) the selection of multiple and appropriate controls to fully enable HCS capabilities and adequate hit selection. Furthermore, the current artificial intelligence (AI) revolution will catalyze further major evolutions in the way HCS is used. These powerful tools are rapidly synergizing with HCS capabilities to bring image-based chemical compound screening into a new dimension. Indeed, the intrinsic data rich characteristics of HCS allows the extraction of a set of features, which include not just shape and spatial metrics but also the intensity and patterning of fluorescently labeled markers, and therefore could be used to define image-based compound fingerprints. Recently, it was demonstrated that image-based compound fingerprints acquired in the context of one HCS campaign could be repurposed to predict compound activity in different customized biological assays and, therefore, dramatically reduce the scale of screens implemented for other projects.

4. ADVANTAGES OF HCS IN COMPARISON TO CURRENT ASSAY METHODS

Cell viability, signaling and transcription as well as disease-related characteristics are the main phenotypic readouts commonly performed in screening of hit compounds. Thus, cell proliferation and/or cell death have been the predominant phenotypic readout using e.g. Alamar® Blue assay, the colorimetric SRB, MTT assays or the ATP content assay [13]. Although these types of readout are productive and informative, they clearly provide only a single information readout per well. Intracellular signaling and transcription assays generally provide a simple link between a complex network of protein interactions and a specific transcription event of interest through pathway driven or active promoter reporter genes such as luciferase and β-lactamase, fluorescent proteins (GFP, YFP), which produces an easily measurable luminescence or fluorescence signal [14]. Although, many single readout assays were used to detect the changes of disease-specific cellular and/or functional phenotypes such as morphological changes, invasion, migration, differentiation or epithelial-to-mesenchymal transition, HCS approaches have obviously become the approach of choice. This trend is exemplified by the ability of HCS to combines automated fluorescence microscopy with quantitative image analysis, allowing the acquisition of unbiased multi-parametric data at the single cell level. HCS has the potential to combine cell viability, cell signaling and transcription, and/or phenotypic readouts in a single well across a large number of conditions in a target-based, phenotypic or combined target-phenotypic drug discovery mode. Therefore, in a single assay, HCS technology has the potential to predict and integrate key biological activities of compounds during the early stages of the drug discovery process [10, 15].

Enzyme-linked immunosorbent assay (ELISA) is designed to capture and quantify the amount of specific proteins or peptides by their epitopes using high affinity antibodies. Typically, the target proteins are either already purified or come from an extract of cells, resulting in the loss of spatial context. Therefore, it is impossible to readily identify which cells had a protein and where it was inside them. Many HCS assays also use antibodies as immunocytochemical affinity tags to label various cellular proteins, but retain the advantage of individual cell measures and subcellular location. Gasparri *et al.* [16] developed a HCS assay for

proliferation of human dermal fibroblasts with fluorescent indicators for brdU incorporation, histone H3 phosphorylation, pRb phosphorylation, and KI-67 expression. Cross-validation by ELISA and flow cytometry uncovered comparatively fewer false-positive/-negative rates with the HCS assay, leading to the assertion that HCS data were inherently of higher quality. In summary, the authors cited higher accuracy of data, both single-cell and population readouts, and the ability to report morphological features as important advantages of the HCS approach. Vogt *et al.* [17] performed a HCS for screening of inhibition activity of ERK dephosphorylation and confirmed the hits by visually inspecting cell images and with standard Western blotting techniques. Analysis of the data showed that this group of compounds was enriched for known cdc25 inhibitors. *In vitro* enzyme assays showed that the ERK inhibitors identified in the HCS inhibited at least one of the DSPases (MKP-3, cdc25B, cdc25A). The authors then performed a multi-parameter high content assay for MKP-3 and ERK phosphorylation in the two subpopulations. They reported a significant measurable difference in phospho-ERK accumulation between the MPK-3 overexpressing cells and the untransfected cells in the same wells. Additionally, the group determined that the compound having the best cellular activity was not one identified as potent in the biochemical screen, suggesting that performing this type of cell-based assay earlier in the drug discovery process is useful.

Flow cytometry or automated cell sorting has likely the most critical advantage when approaching a cell-based assay for the last three decades. However, cell sorting in general does not lend itself to adherent cell lines and there are a very limited number of morphologies that can be measured. Structure-related measurements on a cell are difficult to make due to the flow of the sample. The process is to flow a stream of single cells, passing them through a laser beam to be detected, so it requires the use of large sample volumes and results in high quantities of potentially hazardous wastes. Other considerations for flow cytometry include its high cost, large size, and high maintenance. The greatest advantage of image-based platform HCS is the ability to see and record the biology by means of a picture. Table 1 shows a variety of cell-based methods and how these map to features that are often significant when looking to implementing a cell-based assay [7, 18]. Besides possessing the capability to analyze processes like protein phosphorylation, receptor/ligand interactions, protein expression, cell cycle regulation, enzyme activation or cell proliferation, HCA excels at discerning cell-morphological changes from images of thousands of individual cells, which are generally not traceable by conventional biochemical methods. Morphological changes include intracellular protein translocation, organelle structure changes e.g. in mitochondrial membrane potential, cytoskeletal remodeling, formation of micronuclei or quantification of internalization. This approach then allows for profiling of dose-dependent phenotypic effects induced by different compounds targeting distinct cellular processes. Generally, simultaneous acquisition of data on different properties of cells and their components to allow a more thorough understanding of sample effects is the main advantage of HCS method over other high-throughput methods. An additional advantage of HCS is its flexibility in assay development, e.g. gene expression, protein localization, RNAi screening, G-protein-coupled receptor (GPCR) activation, general signal-transduction and induced pluripotent stem cells (iPSC)-based assays.

Although HCS represents a very attractive approach as described above, designing, performing and interpreting HCS assays is comparatively complicated. Several key elements therefore should be carefully considered. Thus, by multiplexing the readouts extracted from a biological assay, the high-content approach allows the monitoring of not only compound efficacy, but also potential toxicity, screening artifacts, as well as various mechanisms of action associated to treatment. This list of descriptors is usually called phenotypic signature or fingerprint and represents the keystone of HCS but also its weakness, as it could lead to “highly

precise" false positives. A set of principles has been established to facilitate the definition and development of disease-relevant assays, taking advantage of the latest advances in cell-based assay technologies [10, 19]. To select the model of interest, an understanding of the biology at the molecular level, including the cause of the disease of interest, is required. Moreover, many parameters are interdependent, e.g. the cell type determines transfection protocol and experimental timescale. An imaging assay also comprises cell fixation, staining, and microscopy, which must be adapted for the screen. Consequently, Boutros *et al.* [20] suggested that image-analysis steps should be implemented in parallel because this procedure provides direct feedback on the suitability of the assay.

Table 1. Capabilities of variety of cell-based methods [18].

Assay type	Detection mode	Detection method	Intensity-based	Cell-based	Intact cells	Multiplex capability	Cell by cell	Location	Subcelluar structure	Multicellular structure	Assay development to screening
RIA	Scintillation	Scintillation counter	●	◘	x	x	x	x	x	x	●
ELISA	Chemiluminescense	Spectrophotometer/ Luminometer	●	◘	x	◘	x	x	x	x	●
SPA	Scintillation	Scintillation counter	●	◘	x	◘	x	x	x	x	●
Luciferase	Chemiluminescense	Luminometer	●	●	x	◘	x	x	x	x	●
GeneBLAzer®	FRET	Ratiometric fluorescence	●	●	●	◘	◘	x	x	x	●
FLIPR™	Fluorescent	Plate reader	●	●	●	◘	x	x	x	x	●
Flow cytometry	Fluorescent	Multi-laser PMT flow cytometer	●	●	●	●	●	◘	○	◘	○
HCS	Fluorescent	Multispectral fluorescence imager with analysis	●	●	●	●	●	●	●	●	●

● Good capability ◘ Limited capability ○ Poor capability x No capability

4. APPLICATION EXAMPLES OF HCS IN NATURAL PRODUCT RESEARCH

The HCS platform was based on many years of basic biomedical research, including the development of research imaging microscopy, imaging technology, and reagents. The two earliest publications on the application of HCS to drug discovery were studies on the translocation of NF-κB, a key role in inflammatory diseases, induced by interleukin-1 and tumor necrosis factor (TNF-α) and on the internalization of a GFP-tagged G-protein-coupled receptor [21, 22]. In a study by Young *et al.* [23] HCS was performed to array cells treated with a total of 6,547 different compounds, 58 % of which were of natural origin. A total of 36 cytological features were extracted and reduced to 6 significant factors. For instance, 12 of the original features were combined to a single factor nuclear size. Eventually, the top 5 % of the whole screening set of 211 compounds whose induced phenotypic responses were significantly different to the average control phenotypes were identified as hits. 96 % of all hit compounds

with similar structure showed similar phenotypes, such as the aurantimycin A and diperamycin or the glucocorticoids clobetasol-17-propionate and dexamethasone.

One of the most important applications of HCS is in searching for hit compounds with *in vitro* anticancer activity. For this bioactivity, several main subcellular molecules of cancer cells have been usually targeted like cell-cycle arrest, expression of apoptosis-regulating proteins and enzymes (e.g. Bcl-2 family, caspases), phosphoinositide 3-kinase (PI3K) and PI3K/Akt signalling pathway, mitochondria, and microtubule (tubulin), etc. For instance, Caie *et al.* [24] correlated phenotypic drug response with several cancer cell types of different genetic background. HCS was run in a four-wavelength assay with four different cancer cell lines. After segmentation of imaged cells by identifying nuclear and cytoplasmic boundaries, 100 features were extracted. It was found that some compounds, e.g. the microtubule stabilizer epothilone B, induced similar phenotypes across all cell lines tested. In case of the translation inhibitor emetine, phenotypic responses of the cell lines cluster differently, indicating highly sensitive, cell-specific responses against this particular drug. It was assumed that p53 is important for emetine activity, as the phenotypic profile of MCF7-p53 (an MCF7 derivative that expresses p53) was significantly different to drug-sensitive wild-type parent line MCF7-wt. Wei *et al.* [25] investigated effects of Jaspolide B, a novel isomalabaricane-type triterpene isolated from sponge *Jaspis* sp., on human hepatoma cells by HCS assays. In this study, incubation with 0.5 μM of jaspolide B caused time-dependent induction of apoptosis in up to 66.8 % of Bel-7402 cells for 48 h, which was confirmed by the enhancement of mitochondrial masses and cell membrane permeability, and nuclear condensation in Bel-7402 and HepG2 cells. Moreover, jaspolide B arrested cell cycle progression at G1 phase of human hepatoma cells in a dose- and time-dependent manner. In addition, treatment of the compound caused dose-dependent disassembly of microtubule cytoskeleton in Bel-7402 cells at indicated concentrations, and this effect being similar but weaker than that of colchicine, a well-known microtubule-disassembly agent. In another study, 1,000 compounds including many natural products were screened by Perlman *et al.* [26] and a sum of 93 descriptors was extracted from stained cells. For generating compound profiles, the descriptors were plotted as a cumulative distribution and then reduced to a single number that represented the point of maximum difference between the control and treated population. For 61 of the 100 compounds, a strong response was obtained by this analysis. Structurally unrelated compounds sharing a common target showed similar response profiles. For the subset of kinase inhibitors no clustering was observed even in case of overlapping targets, maybe due to a variable inhibition of other kinases. The authors also included three poorly characterized compounds in their profiling approach. One of these, austocystin, clustered together with transcription and translation inhibitors. In this case, HCA correctly assigned a compound to a mode of action class. Austocystin D was later on shown to be activated by CYP-enzymes and to induce DNA damage [27]. Link *et al.* [28] reported the first potent and selective phosphoinositide 3-kinase (PI3K) inhibitor that has been discovered and developed using HCS. Activation of the phosphoinositide 3-kinase (PI3K)/Akt signalling pathway is one the most frequent genetic events in human cancer. A HCS assay that monitored the translocation of the Akt effector protein (FOXO) was employed to screen a collection of 33,992 compounds. Pyrazolopyrimidine derivatives were found to be potent FOXO relocators as well as biochemical inhibitors of PI3Kalpha. A combination of virtual screening and molecular modelling led to the development of a structure-activity relationship for synthesis of ETP-45658, which is a selective PI3K-inhibitor and demonstrates mechanism of action in tumor cell lines and *in vivo* in treated mice.

Interestingly, the publications in 2016 from the Quinn group, AU demonstrating how to couple phenotypic screening against non-immortalized human olfactory neurosphere-derived (hONS) cells (primary cells derived from Parkinson's disease patients) with pure natural products using a HCS system. The first screen covered isolated metabolites from marine sponges (*Jaspis splendens*) to prove that the overall system could function. The following screen then utilized a 500-plus pure compound set from the Nature Bank collection at Griffith University [1, 4, 29, 30]. The results demonstrated that such a HCS produced multiple possibilities for the identification of the interaction(s) with cellular organelles/protein interactions, being limited only by the specificity of the fluorescent probes used to demonstrate the responses. To further demonstrate the potential of such HCS, recently an excellent review covering progression in techniques and technologies from 1997 to the end of 2015 as well as almost all aspects of HCS was published by a research group from the Helmholtz Centre in Braunschweig, DE. In addition, iPSC-based assay can be used in large-scale primary screening to finding drug candidates with regards to molecular targets and/or mechanisms of action. For instance, Sherman & Bang [31] demonstrated the feasibility of high-throughput screening of human iPSC-derived neurons using an image-based high-content approach to screen a collection of 4,421 bioactive compounds. The novel findings are three natural products: 2-methoxy-phenylacryloyl-lupinine, which promoted neurite outgrowth, and two Chinese herbal medicines, the diterpenes, andrographolide and triptolide, which inhibited neurites. Another hit not previously implicated in neuritogenesis was the smooth muscle relaxant alverine citrate, which promoted neurite outgrowth in this report. Moreover, three long-chain saturated fatty acids (FAs) were identified as novel neurite growth-promoting hits. A number of compounds for which activity was opposite to previous reports, e.g. the sodium channel blocker dibucaine identified as neurite outgrowth promoting in this screen, but shown previously to inhibit neurites.

Not least, the HCS could be considered as a key role in the detection of toxicity and classification of the compounds on the basis of cellular injury. Tolosa *et al.* [32] designed an *in vitro* testing approach that is capable of potential liver toxicity, which is a major reason for drug non-approvals and withdrawals. Accordingly, HepG2 human cell line was exposed to 78 different natural- and synthetic chemicals at a range of concentrations (1-1,000 μM) and then analysed with the HCS station scan^R (Olympus). Thus, cell parameters associated with nuclear morphology, plasma membrane integrity, mitochondrial function, intracellular calcium concentration, and oxidative stress, indicative of pre-lethal cytotoxic effects and representative of different mechanisms of toxicity were measured at the single cells level, which allows high-throughput screening. This strategy appears to identify early and late events in the hepatotoxic process and suggests the mechanism(s) implicated in the toxicity of compounds or drugs to thereby classify them according to their degree of injury. As the results, fluoxetine, imipramine, maprotiline, and rotenone may cause a high incidence of liver-cell injury at experimental concentration of 100 μM; whilst hepatotoxicity of moderate injury were detected for e.g. 17α-ethinylestradiol (a synthetic derivative of the natural steroid-hormone estradiol), aflatoxin B1, colchicine, cycloheximide, cyclosporine A, erythromycin, etoposide, simvastatin, and lovastatin.

At INPC-VAST, we have been recently developed the fluorescent image-based HCS towards screening of the anti-cancer, anti-inflammatory activities as well as *in vitro* cytotoxic effects of compounds or drugs with regards to molecular targets, e.g. NF-κB translocation, cell-cycle arrest, early and late-stage apoptosis, mitochondrial changes, and neurotoxicity using mouse hippocampal neuronal-cell model, etc. Acquisition and image analysis of living or fixed cells after treatment with natural- or synthetic chemicals are carried out with a HCS station

scan^R that allows the simultaneous quantification of selected biological targets stained by corresponding fluorescent dyes (Figure 2).

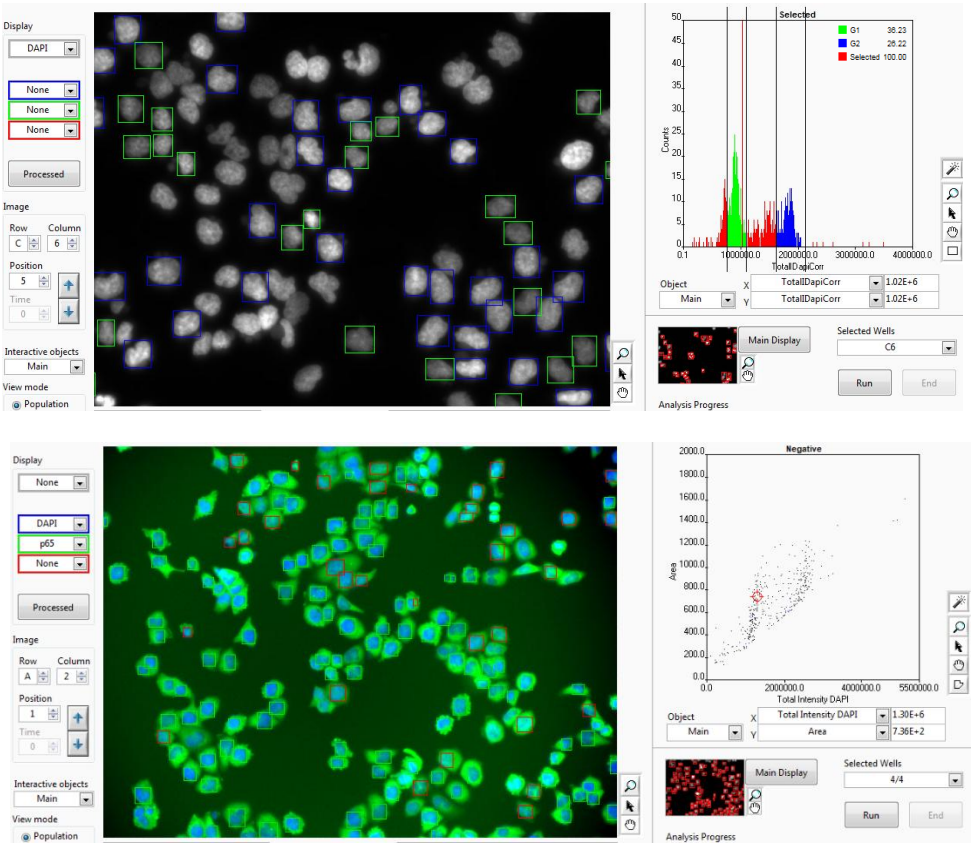

Figure 2. The use of high-content screening station at INPC-VAST for arrested cell-cycle analysis (Hep-G2 cell line; *upper figure*) and NF-κB p65 translocation in HeLa cells (*lower figure*). Cells (10,000 cells/well of a 96-well plate) were stained with DAPI, GFP after treatment with 1-20 µM test compounds. Images were taken with 20x/40x objectives, sCMOS camera (Hamamatsu ORCA-flash 4.0, JP) and analyzed by using a scan^R Analysis ver.2.7.2 software.

5. CONCLUSION

A major bottleneck in the screening of natural products as tools for hit identification in chemical biology research is the identification of their mode of action or targets on individual cell and subcellular level. High-content screening (HCS) has been recognized as an alternative approach to easing these key bottlenecks and plays an increasingly important role in both early and late-stage drug discovery for two decades. It is also expected that *in vitro* toxicology will be the next major area of HCS application in drug development process, which may ultimately predict *in vivo* toxicity and classification of compounds basing on their degree of cellular injury.

Acknowledgements. The support by Vietnam Academy of Science and Technology in the form of equipment for VAST-key lab of bioassay (2015-2018, PI: Prof. Pham Quoc Long) and research funding under grant No. VAST 04.05/18-19 was acknowledged.

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